Non-specific effects of methyl ketone peptide inhibitors of caspases

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Abstract Caspases are a family of cysteine proteases which play a crucial role in apoptosis and inflammation. The involvement of caspases in these processes can be demonstrated by their irreversible inhibition with fluoromethyl ketone and chloromethyl ketone derivatives of peptides resembling the cleavage site of known caspase substrates. These inhibitors irreversibly alkylate the cysteine residue in the active site of caspases. In this study we show that a biotinylated fluoromethyl ketone peptide inhibitor of caspases (z-VAD.fmk) also efficiently affinity-labeled cathepsin B and cathepsin H. In addition, the caspase inhibitors z-VAD.fmk, z-DEVD.fmk and Ac-YVAD.cmk also efficiently inhibited cathepsin B activity in vitro and in tissue culture cells at concentrations that are generally used to demonstrate the involvement of caspases.

Key words: Methyl ketone peptide inhibitor; Caspase; Cathepsin

1. Introduction

In recent years, programmed cell death or apoptosis has been studied intensively. The main 'executioners' of apoptosis seem to be caspases which are cysteine proteases that specifically cleave their substrate after an aspartate residue (for review see [1]). Caspases reside in the cell as inactive proforms, which are proteolytically converted into their active forms by autoprocessing or by other caspases. Active caspases then cleave specific substrates that need to be activated or inactivated during the process of apoptosis [1]. In addition, specific members of the caspase family are also involved in the maturation of proinflammatory cytokines such as prointerleukin-1β (proIL-1β), and are therefore important mediators of inflammation [2].

A helpful tool to elucidate the role of caspases in apoptosis and the maturation of cytokines has been so-called specific caspase peptide inhibitors that are aldehyde or fluoro/chloromethyl ketone derivatives of the target cleavage sequence in the physiological substrates that were first identified. The irreversible caspase-1 inhibitor Ac-YVAD.cmk is based on the target sequence in proIL-1β (YVHD) [3]. Another inhibitor, Ac-DEVD.fmk, is derived from the cleavage site in poly-(ADP-ribose)polymerase (PARP) and inhibits caspase-3 and members of the caspase-3 subfamily [4]. Finally, z-VAD.fmk has been used as a broad spectrum inhibitor of caspases. Such compounds are able to block the release of IL-1β from human monocytes, or to inhibit apoptosis in diverse cell types [5,6]. Caspase inhibitors such as z-VAD.fmk also import protection in several in vivo models of acute liver diseases [7,8]. Finally, biotinylated derivatives of these inhibitors have also been demonstrated to be useful for affinity labeling of active caspases in crude cell extracts [9–11]. In a similar approach to identify active caspases in murine liver extracts, we recently found that the biotinylated caspase inhibitor z-VAD.fmk also labeled cathepsin B [12]. Here we further document the non-specificity of methylketone peptide inhibitors of caspases by showing that biotinylated z-VAD.fmk could efficiently label purified cathepsin B as well as cathepsin H. Moreover, several so-called specific caspase inhibitors were found to strongly inhibit cathepsin B activity in vitro and in cultured cells at concentrations that are commonly used to demonstrate an involvement of caspases.

2. Materials and methods

2.1. Cells and reagents

The murine fibrosarcoma cell line WEHI164 (obtained from Dr. T. Espevik, University of Trondheim, Norway) was maintained in RPMI 1640 supplemented with 10% fetal calf serum. Recombinant murine TNF was produced by *Escherichia coli* and purified to at least 99% homogeneity. The preparation used had a specific activity of 1.4×10^8 IU/mg, as determined with the international standard (code 88/532; National Institute for Biological Standards and Control, Potters Bar, UK). Recombinant murine caspase-1 and caspase-3 were produced in *E. coli* and have been described previously [12]. Purified cathepsins B, D and H from human liver, as well as z-FA.fmk and Ac-YVAD.cmk were obtained from Calbiochem-Novabiochem International (San Diego, CA, USA). z-DEVD.fmk, z-VAD.fmk, biotinylated z-VAD.fmk, and z-ARR.AFC were supplied by Enzyme Systems Products (Dublin, CA, USA). Ac-DEVD.AMC and Ac-YVAD.AMC were from Peptide Institute (Osaka, Japan).

2.2. Affinity labeling with biotinylated z-VAD.fmk

500 ng purified cathepsins or caspase-1 were incubated with 1 μM biotinylated z-VAD.fmk for 20 min at 30°C in a total volume of 25 μl cell free system buffer (10 mM HEPES-NaOH pH 7.4, 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH_2PO_4, 0.5 mM EGTA, 2 mM $MgCl_2$, 5 mM pyruvate, 0.1 mM PMSF and 1 mM dithiothreitol). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes which were then incubated with streptavidin-conjugated horseradish peroxidase (1/500). The affinity-labeled proteins were visualized by ECL (Amersham Life Science, Amersham, UK).

2.3. Measurement of caspase and cathepsin B activity

25 ng purified caspases or cathepsin B were incubated for 1 h at 30°C in cell free system buffer with 50 μM of the fluorogenic caspase substrates Ac-DEVD.AMC or Ac-YVAD.AMC, or the fluorogenic cathepsin B substrate z-ARR.AFC, respectively. In the case of inhibitor studies, enzymes were preincubated for 30 min at room temper-

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Abbreviations: Ac-DEVD.AMC, acetyl-Asp-Glu-Val-Asp-AMC; Ac-YVAD.cmk, acetyl-Tyr-Val-Ala-Asp.cmk; AFC, 7-amino-4-trifluoromethyl coumarin; AMC, 7-amino-4-methyl coumarin; cmk, chloromethyl ketone; DMSO, dimethylsulfoxide; fmk, fluoromethyl ketone; IL-1, interleukin-1; PMSF, phenylmethylsulfonyl fluoride; TNF, tumor necrosis factor; z-VAD.fmk, benzyloxycarbonyl-Val-Ala-Asp.fmk; z-FA.fmk, benzyloxycarbonyl-Phe-Ala-fmk; z-ARR.AFC, benzyloxycarbonyl-Ala-Arg-Arg-AFC

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ature with a serial dilution of the inhibitors. The release of 7-amino-4-methyl coumarin (AMC) was continuously monitored during 60 min in a fluorometer (CytoFluor; PerSeptive Biosystems, Cambridge, MA, USA) at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Free 7-amino-4-trifluoromethyl coumarin (AFC) was monitored at an excitation wavelength of 409 nm and an emission wavelength of 505 nm. Data are expressed as increase in fluorescence as a function of time (ΔF/min). To determine cathepsin B activity in total cell extracts, cells were lysed in 10 mM Tris-HCl pH 7, 1% NP-40, 200 mM NaCl, 5 mM EDTA, 1 mM PMSF. Cleared cell extracts (25 μg cellular protein) were incubated for 1 h at 30°C in cell free system buffer with 50 μM of the fluorogenic cathepsin B substrate z-ARR.AFC as described above.

2.4. Apoptosis assay

WEHI164 cells were seeded at 3×10^5 cells/ml in a 6-well plate in a total volume of 5 ml. Cells were stimulated with TNF (1000 IU/ml) for 6 h in the presence of actinomycin D (1 μg/ml). The latter was added to enhance the TNF sensitivity of the cells as described previously [13]. At the end of the incubation period, adherent cells were trypsinized and combined with cells that were freely floating in the medium. Cell death was determined by trypan blue staining.

3. Results

3.1. Affinity labeling of cathepsins with biotinylated z-VAD.fmk

Affinity labeling with biotinylated irreversible caspase inhibitors has been shown to be useful for detecting active caspases in cell extracts by means of Western blotting [9–11]. Active caspases can then be visualized on a Western blot with streptavidin-conjugated peroxidase. In a similar approach with the biotinylated caspase inhibitor z-VAD.fmk and murine liver extracts, we could recently demonstrate the specific labeling of a number of proteins that could be visualized as four discrete bands [12]. Surprisingly, upon microsequencing of these labeled proteins they turned out to correspond to cathepsins B and H. These proteases are totally unrelated to caspases, except for being both cysteine proteases. Because the cathepsin family consists not only of cysteine proteases, but also of serine and aspartic acid proteases, we analyzed whether biotinylated z-VAD.fmk could also label the serine protease cathepsin D. Therefore, purified cathepsins B, H and D were incubated with 1 μM biotinylated z-VAD.fmk and analyzed for binding by SDS-PAGE and Western blotting. Purified caspase-1 was included as a positive control. As expected, both caspase-1 and the cysteine proteases cathepsins B and H were clearly labeled with biotinylated z-VAD.fmk (Fig. 1, upper panel). In contrast, labeling of the serine protease cathepsin D could not be detected. According to their size and the corresponding Coomassie blue gel staining of the purified preparations (Fig. 1, lower panel), the labeled bands are likely to correspond to the p20 subunit of caspase-1 (indicated as 1) and specific processing products of cathepsins. This was further confirmed by immunoblot detection with specific antibodies raised against caspase-1 and cathepsin B, respectively (data not shown). In the case of cathepsin B, z-VAD.fmk specifically labeled the intermediate form of cathepsin B lacking its prodomain (indicated as 5), whereas in the case of cathepsin H specific labeling of the heavy chain was observed (indicated as 9). The lower migrating bands which are not labeled by biotinylated z-VAD.fmk correspond to further processing products of cathepsins as indicated in the legend to Fig. 1. The above results clearly demonstrate non-specific binding of the caspase inhibitor z-VAD.fmk to non-related cysteine proteases belonging to the cathepsin family.

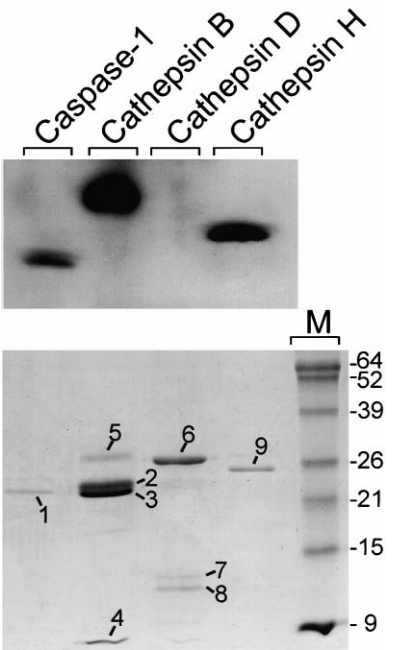

Fig. 1. Affinity labeling of cathepsins with biotinylated z-VAD.fmk (upper panel) and Coomassie blue gel staining of the purified proteases used for labeling (lower panel). 500 ng purified caspase-1, cathepsin B, cathepsin D, or cathepsin H were incubated with 1 μM biotin-VAD.fmk. Proteins were separated by SDS-PAGE and affinity-labeled proteins were revealed as described in Section 2. For Coomassie blue detection, 4 μg of each protease was loaded on a separate gel. In the case of caspase-1 the labeled polypeptide corresponds to the p20 subunit (band 1 in the lower panel), whereas the labeled cathepsin polypeptides correspond to cathepsin B without prodomain (band 5 in the lower panel) and the heavy chain of cathepsin H (band 9 in the lower panel), respectively. The other Coomassie blue-stained bands that are not labeled correspond to the heavy chain of cathepsin B with and without a short linker region (bands 2 and 3), the light chain of cathepsin B (band 4), and the heavy (band 6) and light chains with or without a linker region (bands 7 and 8) of cathepsin D. The light chain of cathepsin H runs in the migration front of the gel and is therefore not detectable.

3.2. Inhibition of cathepsin B by caspase peptide inhibitors

The aspecific labeling of cathepsins B and H with biotinylated z-VAD.fmk prompted us to analyze whether this binding also led to an inhibition of enzyme activity. Therefore, purified cathepsin B was preincubated with 1 μM z-VAD.fmk and analyzed for cathepsin B activity on the fluorogenic substrate z-ARR.AFC. The cathepsin B inhibitor z-FA.fmk was used as a positive control. Indeed, z-VAD.fmk completely inhibited the activity of cathepsin B (Fig. 2A). Similarly, preincubation with z-DEVD.fmk, which has been generally used as a specific inhibitor of caspases of the caspase-3 subfamily, led to a complete inhibition of cathepsin B activity. In contrast, preincubation with the caspase-1 inhibitor Ac-YVAD.cmk had no effect. However, a partial inhibition of cathepsin B activity could be observed at 100 μM (data not shown). Our results clearly demonstrate that methyl ketone peptide inhibitors of caspases, at concentrations which are generally used to demonstrate the involvement of caspases, can also inhibit cathepsin B.

To further demonstrate this non-specific inhibition of cathepsins by methyl ketone inhibitors of caspases, we analyzed

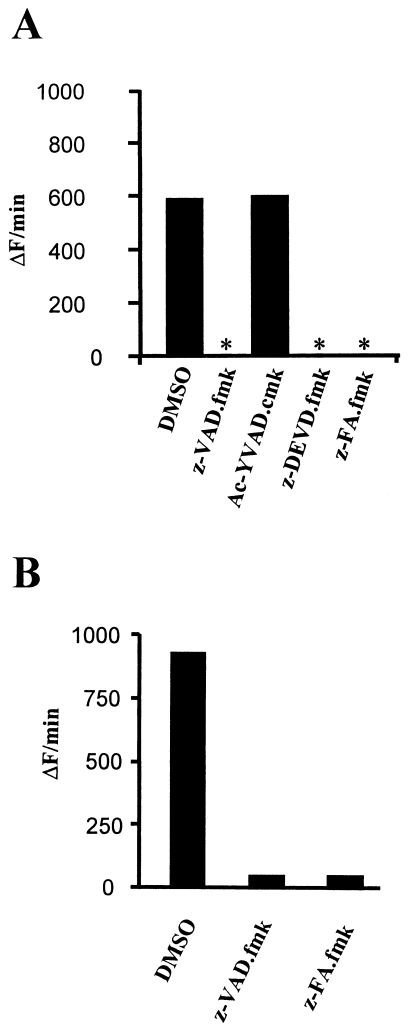

Fig. 2. Effect of chloro/fluoromethyl ketone peptide inhibitors on the activity of cathepsin B in vitro and in tissue culture cells. A: Purified cathepsin B (25 ng) was preincubated for 30 min with the indicated inhibitors (1 μM), and its activity was measured with the fluorogenic substrate z-ARR.AFC as described in Section 2. Data are expressed as increase in fluorescence as a function of time (Δ*F*/min). DMSO which was used as a solvent for the inhibitors served as a negative control. Results are representative of at least three independent experiments with standard deviations less than 10% ($n = 3$). An asterisk means that there was no detectable activity present. B: WEHI164 cells were treated for 6 h with the indicated inhibitors (50 μM), and cathepsin B activity in cell extracts was measured as described in A. Results are representative of three independent experiments with standard deviations less than 10% ($n = 3$).

the effect of z-VAD.fmk on cathepsin B activity in WEHI164 cells. We used an inhibitor concentration of 50 μM because this concentration has been used previously in many cellular studies investigating the role of caspases. Pretreatment of WEHI164 cells with the cathepsin B inhibitor z-FA.fmk as well as with the caspase inhibitor z-VAD.fmk completely inhibited cathepsin B activity that could be measured in cell extracts with the fluorogenic substrate z-ARR.AFC (Fig. 2B). Comparable results were obtained with Ac-DEVD.fmk and when analyzed on several other cell lines (data not shown). Therefore, we can conclude that treatment of cells with so-called specific caspase inhibitors at concentrations that have been commonly used might also non-specifically inhibit cathepsin B and related proteases.

3.3. Caspase peptide substrates are not cleaved by cathepsin B

Similar to the processing of the fluorogenic substrate z-ARR.AFC by cathepsin B, caspase activity can be measured on fluorogenic substrates such as Ac-YVAD.AMC and Ac-DEVD.AMC. In view of the above described inhibition of cathepsin B with the corresponding methyl ketone peptide inhibitors, we tested whether purified cathepsin B had any activity on these substrates. As a positive control we analyzed their processing by purified caspase-1 and caspase-3, while the activity of cathepsin B was also tested on z-ARR.AFC. As expected, caspase-1 preferentially cleaved Ac-YVAD.AMC, whereas caspase-3 specifically cleaved Ac-DEVD.AMC (Fig. 3). In addition, cathepsin B was clearly active on z-ARR.AFC, but did not cleave significant amounts of the caspase substrates. Thus, although cathepsin B can be fully inhibited by z-DEVD.fmk, it cannot cleave the corresponding substrate Ac-DEVD.AMC to a significant level.

3.4. The protective effect of z-VAD.fmk on TNF-induced apoptosis of WEHI164 cells is not due to cathepsin B inhibition

Pretreatment of cells with the caspase inhibitor z-VAD.fmk or z-DEVD.fmk can protect many cell lines against TNF-induced apoptosis [14,15]. The most likely explanation for this is of course the inhibition of caspases that are involved in the apoptotic process. However, in view of the non-specific inhibition of cathepsin B by these inhibitors one cannot exclude a role for cathepsin B as well. The more because cathepsin B has already been implicated in some apoptotic models and in a number of inflammatory diseases [16,17]. Therefore we compared the effect of pretreatment of the cells with 50 μM of the caspase inhibitor z-VAD.fmk or the cathepsin B inhibitor z-FA.fmk on TNF-induced apoptosis of WEHI164 cells. Control experiments demonstrated that cathepsin B activity was completely inhibited under these conditions (Fig. 2A). Co-treatment with the transcription inhibitor actinomycin D was used as a TNF-sensitizing agent [13]. Whereas z-

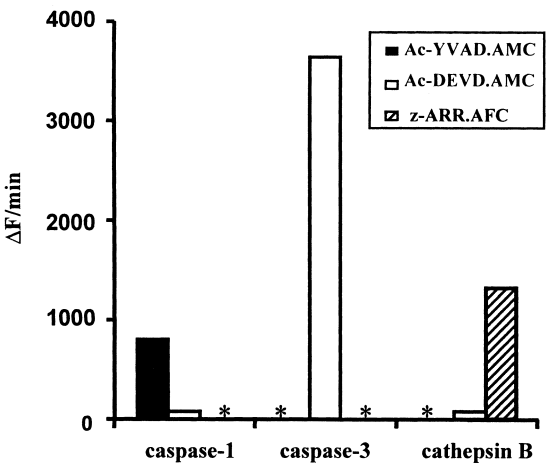

Fig. 3. Effect of purified caspases and cathepsin B on fluorogenic peptide substrates. 25 ng purified caspase-1, caspase-3, or cathepsin B were analysed for their proteolytic activity on Ac-YVAD.AMC, Ac-DEVD.AMC, and z-ARR.AFC as described in Section 2. The increase in fluorescence as a function of time (Δ*F*/min) was used as a parameter for the amount of AMC or AFC that was released. Results are representative of multiple experiments with standard deviations less than 10% ($n = 3$). An asterisk means that there was no detectable activity present.

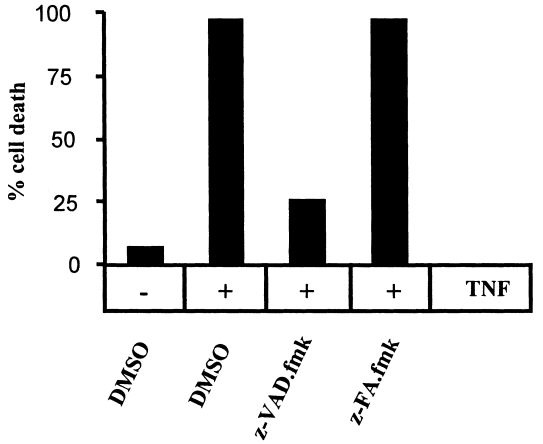

Fig. 4. Comparison of the effect of z-VAD.fmk and z-FA.fmk on TNF-induced apoptosis of WEHI164 cells. Cells were pretreated for 1 h with 50 μM z-VAD.fmk or z-FA.fmk, and subsequently treated for 6 h with 1000 IU/ml TNF in the presence of 1 μg/ml actinomycine D. DMSO which was used as a solvent for the inhibitors served as a negative control. Cell death was analyzed by counting the number of trypan blue positive cells.

VAD.fmk considerably protected the cells from TNF-induced cell death, z-FA.fmk had no effect (Fig. 4). Similar results were obtained with the T cell hybridoma cell line PC60 (data not shown). These results demonstrate that, at least in the cells studied, z-VAD.fmk does not prevent TNF-induced apoptosis by inhibiting cathepsin B.

4. Discussion

Chloro/fluoromethyl ketone peptide inhibitors of caspases are being widely used in experiments that measure cytokine release or apoptosis [1,2,14,18]. Compounds such as Ac-YVAD.cmk, z-DEVD-fmk or z-VAD.fmk are believed to be specific inhibitors of caspases, but their inhibition potential is different for several members of the caspase family. In the present paper we show that they can also potently bind and inhibit cysteine proteases of the cathepsin family. Most important, this already occurs at concentrations that are commonly used to reveal a role of caspases. Indeed, inhibitor concentrations of 50 μM, which completely inhibit cathepsin B, have been used in many cellular studies on apoptosis or inflammation [14,15,19,20]. The reason for this non-specific inhibition is still unclear. The secondary and quaternary structures of caspases do not resemble those of cathepsin B [21,22]. In addition, the active site catalytic machinery of caspases consists of a cysteine-histidine catalytic dyad, which is different from the cysteine-histidine-asparagine triad of cathepsin B. However, the catalytic cysteine residue thiolate is an activated nucleophile which is known to be very reactive with fmk and cmk groups [23]. Nevertheless, inhibition of cathepsin B with chloro/fluoromethyl ketone peptide inhibitors is not solely mediated by the reactivity of the methyl ketone group, as no inhibitory effect could be observed with the related inhibitor z-AAD.fmk (data not shown). Therefore, the peptide part of the inhibitor also seems to be involved in the inhibition of cathepsin B. In contrast to caspases, which require an aspartic acid residue at the P1 site, cathepsin B is known to have a much broader substrate specificity. This flexibility might also explain its sensitivity to caspase inhibitors as has been observed in our study. Whatever the reason for the non-specific inhibition of cathepsin B by these caspase inhibitors might be, our results imply that inhibitors such as z-VAD.fmk, z-DEVD.fmk or Ac-YVAD.cmk should be used with care. The fact that cathepsin B has already been implicated in some apoptotic models and in a number of inflammatory diseases [16,17] might complicate the interpretation of results obtained with so-called specific caspase inhibitors. Indeed, a recent report suggested caspases as apical activators of cathepsin B, based on the inhibition of bile salt-induced cathepsin B activity with the caspase inhibitors z-VAD.fmk and z-DEVD.fmk [24]. In addition, the use of affinity labeling with biotinylated methyl ketone inhibitors as a technique to isolate active caspases from cell extracts might be hampered by the binding of cathepsin B or related proteases. Finally, the inhibition of a housekeeping protease such as cathepsin B might also limit the therapeutic use of fmk- and cmk-derived caspase inhibitors in different diseases.

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